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EPIDEMIOLOGY AND SURVEILLANCE

Absence of a High Level of Duplication of the Plasmepsin II Gene in Africa

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ABSTRACT Resistance to piperaquine has been associated with the amplification of the plasmepsin II gene in Cambodia. None of the 175 African isolates that we analyzed had plasmepsin II gene amplification (piperaquine 50% inhibitory concentration ranged from 0.94 to 137.5 nM), suggesting there is a low prevalence of piperaquine reduced susceptibility in Africa. Additionally, the few isolates with reduced susceptibility to piperaquine did not harbor amplification of the plasmepsin II gene.

KEYWORDS malaria, *Plasmodium falciparum*, antimalarial drug, resistance, *in vitro*, molecular marker, plasmepsin II gene, piperaquine

Plasmodium falciparum resistance to most antimalarial drugs has emerged in Southeast Asia and spread to Africa (1, 2). Since 2005, the World Health Organization (WHO) recommended artemisinin-based combination therapies (ACT) as the first-line treatment for uncomplicated malaria, followed by artesunate for the treatment of severe malaria. However, artemisinin derivative-resistant *P. falciparum* strains emerged in western Cambodia, Myanmar, and Thailand, and eventually in all of Southeast Asia (3, 4). As soon as the last marketed ACT, dihydroartemisinin-piperaquine, was used, resistance emerged in Cambodia and later in Vietnam (5–8). In this context, it is essential to have markers of resistance to monitor the emergence and spread of resistance to dihydroartemisinin-piperaquine. Mutations (Y493H, F446I, and C580Y) in the propeller domain of the kelch 13 (K13) gene (PF3D71343700) were associated with *in vivo* and *in vitro* resistance to artemisinin in Southeast Asia (9, 10). Two recent studies showed that *in vitro* and *in vivo* resistance to piperaquine were associated with the amplification of the copy number of the plasmepsin II gene (PF3D7_1408000) (11, 12). However, these data were validated only on Cambodian isolates. The objective of the present study was to evaluate the copy numbers of the gene in African *P. falciparum* isolates and the gene's association with *in vitro* susceptibility to piperaquine.

A total of 175 *P. falciparum* isolates were successfully evaluated for the copy number of the plasmepsin II gene and assessed for *ex vivo* susceptibility to piperaquine. The isolates were collected from patients hospitalized in France from January 2015 to April 2017 with imported malaria from a country where malaria is endemic and, more particularly, from African French-speaking countries, such as Côte d'Ivoire, Cameroon, the Central African Republic, the Republic of the Congo, Guinea, Burkina Faso, Togo, Gabon, and Senegal (Table 1). The samples were sent from different civilian or military hospitals of the French National Reference Center for Imported Malaria Network (Aix en Provence, Bordeaux, Marseille, Montpellier, Nice, Toulon, and Toulouse) to the French

Received 23 February 2018 **Returned for modification** 5 April 2018 **Accepted** 16 August 2018

Accepted manuscript posted online 4 September 2018

Citation Robert MG, Foguim Tsombeng F, Gendrot M, Mosnier J, Amalvict R, Benoit N, Torrentino-Madamet M, Pradines B, the French National Reference Centre for Imported Malaria Study Group. 2018. Absence of a high level of duplication of the plasmepsin II gene in Africa. Antimicrob Agents Chemother 62:e00374-18. https://doi.org/10.1128/AAC.00374-18.

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TABLE 1 Geographical repartition of the 175 *Plasmodium falciparum* isolates

Country	Isolate no.	Percentage
Cameroon	47	26.9
Côte d'Ivoire	37	21.1
Central African Republic	18	10.3
Guinea	15	8.6
Republic of the Congo	14	8.0
Togo	8	4.6
Burkina Faso	7	4.0
Gabon	7	4.0
Benin	5	2.9
Chad	2	1.1
Comores	2	1.1
Ghana	1	0.6
Madagascar	2	1.1
Mali	2	1.1
Nigeria	2	1.1
Angola	1	0.6
Democratic Republic of the Congo	1	0.6
Djibouti	1	0.6
Senegal	1	0.6

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Biobanking of human clinical samples used for malaria diagnostics and their secondary uses for scientific purposes are possible as long as the corresponding patients are informed and have not indicated any objections. This requirement was fulfilled here by giving verbal information to the patients, and no immediate or delayed patient opposition was reported to the hospital clinicians. Informed consent was not required for this study because the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

The parasitemia, which ranged from 0.005% to 9.5%, was estimated on thin blood smears that were stained by eosin and methylene blue using a RAL kit (Réactifs RAL, Paris, France). The diagnosis of *P. falciparum* monoinfection was confirmed by real-time PCR (LightCycler 2.0; Roche Group, Basel, Switzerland), as previously described (13).

Piperaquine (PPQ) for the *ex vivo* drug susceptibility assay was obtained from Shin Poong Pharm Co. (Seoul, South Korea). PPQ was first dissolved in methanol and later diluted in water to final concentrations that ranged from 1.9 to 998 nM. The isolates were incubated for 72 h in a controlled atmosphere set at 85% N_2, 10% O_2, 5% CO_2, and 37°C (maximum final parasitemia at 0.5% and final hematocrit of 1.5%). The isolates with parasitemia above 0.5% were diluted to parasitemia of 0.5% with fresh, uncontaminated erythrocytes. The drug susceptibility assay was revealed by the HRP2 enzyme-linked immunosorbent assay (ELISA)-based assay implemented in the Malaria Ag complement-enzyme linked immuno sorbent assay (CELISA) kit (reference KM2159; Cellabs Pty. Ltd., Brookvale, Australia) as previously described (14). The 50% inhibitory concentrations (IC_{50}s) were validated only if the optical density (OD) ratio (OD at zero concentration/OD at maximum concentration of drug) was above 1.6 and the 95% confidence interval of the IC_{50} estimation was below 2.0. Each batch of plates was validated on the chloroquine (CQ)-resistant W2 clone of the Indochina strain (obtained from the Malaria Research and Reference Reagent Resource Center [MR4], VA, USA) in four independent experiments, using the same conditions as described below. The mean PPQ 50% inhibitory concentration values for the chloroquine-resistant W2 clone for the different batches used during the study was 54.1 $\pm$ 5.4 nM. There were no significant differences in the responses of the strains to PPQ between the different batches ($P = 0.770$).

The plasmepsin II gene copy number was estimated by TaqMan real-time PCR (LightCycler 2.0, Roche) using the single-copy β-tubulin gene (PF10_0084) as the control housekeeping gene. The following primers and probes were used: 5′-GGA GAT

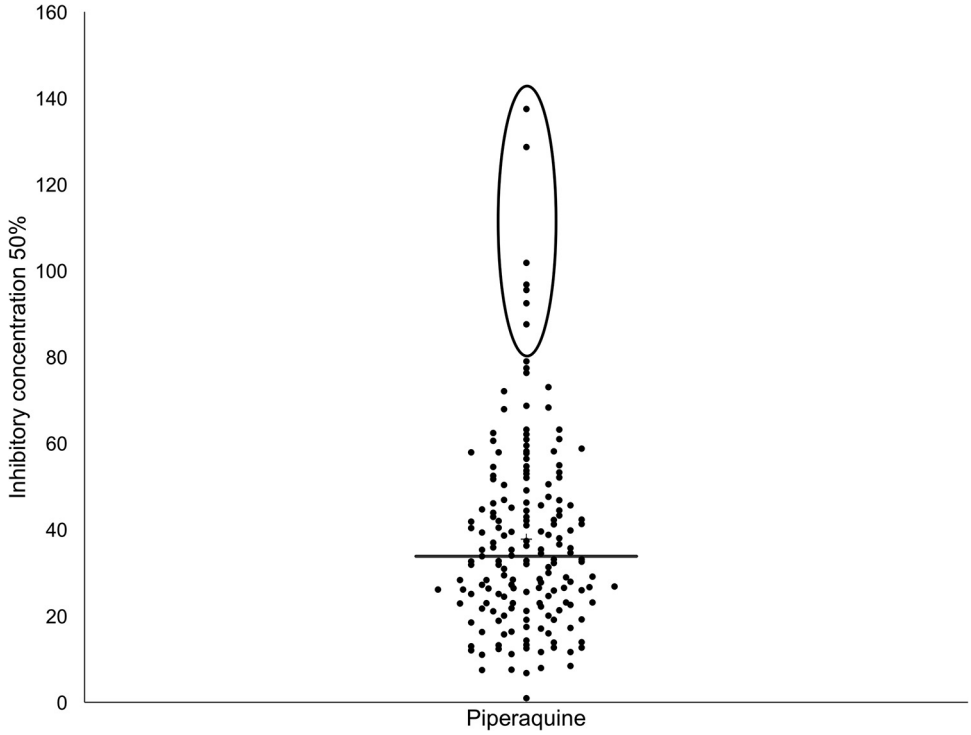

FIG 1 Distribution of *ex vivo* responses (IC_{50}) of 175 African *Plasmodium falciparum* isolates to piperaquine.

AAC CAA CAA CCA TTT AC-3′, 5′-GTT GTA CAT TTA ACA CTT GGG A-3′ and 5′-FAM-CCC ATA AAT TAG CAG ATC CTG TAT C-TAMRA-3′ for the plasmepsin II gene, and 5′-TGA TGT GCG CAA GTG ATC C-3′, 5′-TCC TTT GTG GAC ATT CTT CCT C-3′ and 5′-FAM-TAG CAC ATG CCG TTA AAT ATC TTC CAT GTC T-TAMRA-3′ for the β-tubulin gene (Eurogentec, Angers, France). PCRs were carried out using a 1× LightCycler TaqMan master mix (Roche, Germany), 900 nM forward primer, 900 nM reverse primer, 250 nM TaqMan probe, and 3 μl of template DNA. The thermal cycling conditions were 45 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in duplicate. The $2^{-\Delta\Delta CT}$ method of relative quantification (where C_T indicates cycle threshold) was used and adapted to estimate the copy number of the plasmepsin II gene, using the formula $\Delta\Delta C_T = (C_{T\text{plasmepsin II}} - C_{T\beta}\text{-tubulin})_{\text{sample}} - (C_{T\text{plasmepsin II}} - C_{T\beta}\text{-tubulin})_{\text{calibrator}}$. Genomic DNA extracted from the *P. falciparum* 3D7 strain, which has a single copy of each gene, was used as the calibrator, and the β-tubulin gene served as the control housekeeping gene in all experiments. We previously verified that the PCR efficiency was identical for the two genes. Samples were evaluated twice if the C_T standard deviation was above 0.5 and if the copy number was above 1.5. Isolates with a copy number of $\geq$1.6 were classified as isolates with 2 copies (11, 15). A control parasite isolate harboring at least two copies of the plasmepsin II gene and sampled from a patient with malaria after returning from Cambodia was used to validate the quantitative PCR assay (sample kindly provided by Sandrine Houzé).

The *ex vivo* IC_{50} curves of PPQ were satisfactorily fitted to a sigmoidal function. Unlike the cultured-adapted Cambodian parasites (16), none of our isolates exhibited a bimodal dose-response curve when exposed to PPQ. The PPQ IC_{50} of the 175 isolates ranged from 0.94 to 137.5 nM (Fig. 1). A wide range of the PPQ responses was already observed in several studies, regardless of the methodology used. PPQ IC_{50}s, assessed by a 72 h incubation with atmospheric generators for capnophilic bacteria and HRP2 ELISA in isolates from Dakar in 2013 to 2014 and 2013 to 2015, ranged from 2.5 to 168 nM and 3.9 to 241.9 nM, respectively (17, 18). The distribution of PPQ IC_{50} from 313 isolates obtained between 2008 and 2012 from patients hospitalized in France for imported malaria and assessed by a 42-h isotopic test ranged from 9.8 to 217.3 nM (19). A wide

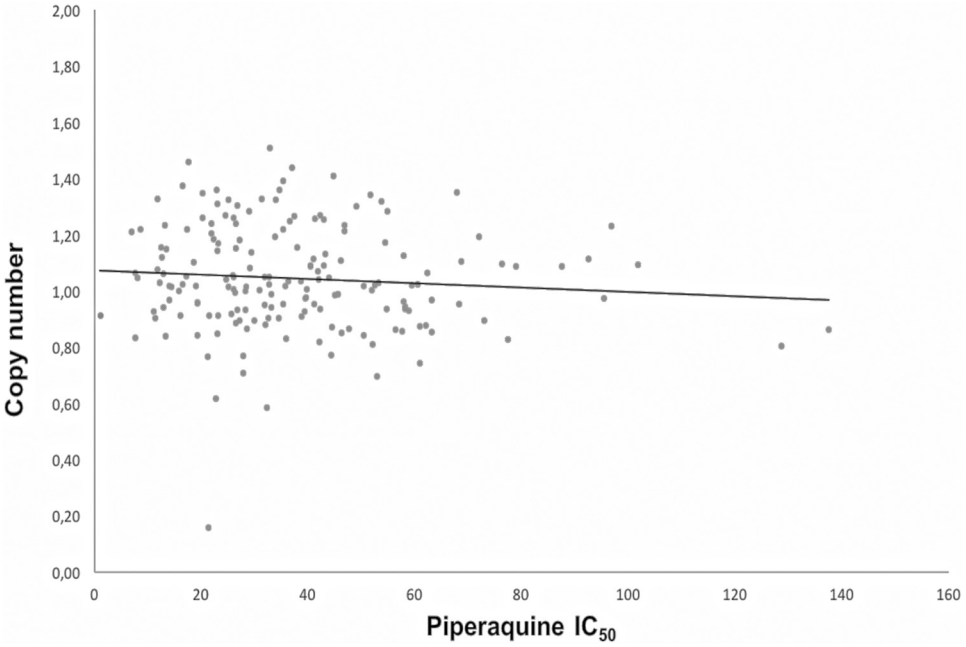

FIG 2 Copy number of the plasmepsin II gene as a function of piperaquine IC_{50} of 175 African *Plasmodium falciparum* isolates.

range of PPQ responses (3.1 to 188.9 nM) was also observed in isolates collected in 2010 to 2013 in Uganda and assessed using HRP2 ELISA detection (20). Only the isolates collected in 2016 in Uganda and assessed using a 72 h fluorescence assay with SYBR green I detection presented a narrow range for PPQ IC_{50}, from 1.8 to 26.6 nM (21). In the absence of standardized *ex vivo* and *in vitro* tests, it is difficult to compare data from different laboratories. IC_{50} and cutoff values for *in vitro* resistance are specific to the methodology. The *in vitro* effects and the IC_{50}s for antimalarial drugs depend on incubation and gas conditions and methodology (22–25). The isolates collected in Uganda in 2016 and assessed using a 72-h fluorescence assay with SYBR green I detection showed a narrow range for PPQ IC_{50}, unlike those collected in 2010 to 2013 and assessed using HRP2 ELISA, which presented a wide range of PPQ responses (20, 21). Only the isolates that were assessed with SYBR green I detection showed a narrow range for PPQ IC_{50}. Another hypothesis for a source of variation in drug responses may be the storage and the time between sample collection from patients and the completion of the *ex vivo* test. Blood was collected before therapy in an EDTA tube. Drug susceptibilities were assessed immediately or from samples stored at 4°C, even during transport, for a maximum of 48 h and without short-term culture. After collection from patients, the storage temperature of the samples was controlled by sensor. Additionally, Senegalese isolates, which were assessed immediately, presented the same wide range of *in vitro* responses (17, 18). One isolate had a reduced susceptibility to PPQ (IC_{50}, >135 nM) (19). Another possible source of variation in the PPQ drug responses could result from the range of parasitemias used in the assay. This is because samples below 0.5% parasitemia were included in the assays and thus could not be adjusted to the standardized assay parasitemia.

Significant cross-susceptibilities were found between PPQ and mefloquine (coefficient of correlation [r] = 0.453; $P < 0.0001$), pyronaridine ($r = 0.406$; $P < 0.0001$), quinine ($r = 0.247$; $P = 0.0011$), and monodesethylamodiaquine ($r = 0.189$; $P = 0.0138$). Associations between PPQ and chloroquine or lumefantrine were not significant ($r = 0.137$; $P = 0.0755$; and and $r = 0.114$; $P = 0.1413$, respectively). The values of the coefficient of determination (r^2) of the different associations were too low to explain the wide range of PPQ responses.

The copy number values ranged from 0.16 to 1.51 with a mean of 1.04 (Fig. 2). None

of the isolates had more than one copy of the plasmepsin II gene.

We did not observe any plasmepsin II gene amplification in the African isolates analyzed in this study. There was no association between the copy number of the plasmepsin II gene and susceptibility to PPQ. The most representative countries in terms of numbers were Cameroon and Côte d'Ivoire (47 and 37 isolates, respectively). Seven isolates (IC_{50}, >90 nM) were outside the main scatter points representing the distribution of the *in vitro* responses to PPQ (Fig. 1) and could be considered parasites with reduced susceptibility to PPQ, although below 135 nM for six of them. The Cambodian *P. falciparum* strains, which were resistant *ex vivo* to PPQ and showed amplification of the plasmepsin II gene, presented IC_{50} values ranging from 89.3 to 159.6 nM (12). Among these seven isolates, four came from Cameroon and two from Côte d'Ivoire, suggesting the presence of few *P. falciparum* strains with reduced susceptibility to PPQ. The primary limitation of the present study is the low number of parasite isolates with reduced susceptibility to PPQ, probably due to the low use of dihydroartemisinin-piperaquine in Africa compared to that in Southeast Asia. Additionally, none of the seven isolates with reduced susceptibility to PPQ (IC_{50}, >90 nM) harbored more than one copy of the plasmepsin II gene. This phenomenon was also shown in Cambodian isolates harboring a single copy of the plasmepsin II gene, for which a large range of PPQ IC_{50} was observed, as well as in some parasites with high IC_{50} values (12). The use of the standard *ex vivo* assay and not the PPQ survival assay (PSA) can be questionable (11, 26). However, Amato et al. identified the same association between *ex vivo* PPQ resistance and the amplification of the plasmepsin II gene by using a standard susceptibility drug assay (12). The resistant parasites showed *ex vivo* IC_{50}s between 89.3 and 159.6 nM and *in vitro* IC_{50}s between 55.9 and 79.7 nM after cultures had adapted, reflecting a high PSA survival % that ranged from 52.2 to 74.9% (12). Additionally, a novel mutation (F145I) on the *P. falciparum* chloroquine-resistance transporter gene (*pfcrt*) was recently identified and described as having an association with the PPQ IC_{90} values in Cambodian isolates by the standard *in vitro* assay (27). The influence of this mutation will have to be evaluated in African *P. falciparum* isolates.

The amplification of the plasmepsin II gene does not fully explain the decreased *in vitro* susceptibility to PPQ of some *P. falciparum* parasites in Africa. Indeed, no correlation was observed between the copy number of the plasmepsin II gene and *ex vivo* susceptibility to piperaquine in Ugandan *P. falciparum* isolates (21). The use of dihydroartemisinin-piperaquine as intermittent preventive treatment during pregnancy (IPTp) did not select for genotypes associated with amplification of the plasmepsin II gene in Uganda (28). Additionally, no amplification of the plasmepsin II gene was found in Cameroonian recrudescent *Plasmodium falciparum* parasites 2 years after treatment by dihydroartemisinin-piperaquine (29). Gupta et al. reported that 1.1% of the *Plasmodium falciparum* isolates circulating in Mozambique in 2015 harbored multiple copies of the plasmepsin II gene (30). However, none of these isolates were compared with *in vitro* data or clinical responses. These data do not allow assessment of the possible association between amplification of the plasmepsin II gene and reduced susceptibility to PPQ. Additionally, Amato et al. showed that more than half of isolates harboring multiple copies of the plasmepsin II gene presented low susceptibility to PPQ, suggesting that amplification of the plasmepsin II gene did not alone explain reduced susceptibility to PPQ (12).

These finding suggest that copy number variation of the plasmepsin II gene may not alone predict PPQ resistance in Africa. It seems that other resistance mechanisms and therefore other molecular markers may exist in Africa compared to those in Asia. This phenomenon has also been observed with resistance to artemisinin, which is associated with *pfk13* polymorphisms in Asia, while no polymorphism is observed in most cases of clinical failure of ACT in Africa (10, 31–35). To overcome the limitation of this study, i.e., the low number of samples with reduced susceptibility, it is imperative to further assess more isolates from different geographical areas of Africa, and especially more *P. falciparum* strains resistant to PPQ from Africa.

ACKNOWLEDGMENTS

We thank the patients and the staff of the hospitals of the French National Reference Center for Imported Malaria Network. We thank Sandrine Houzé (Centre national de reference du Paludisme, Paris, France) for providing the clinical sample with two copies of the plasmepsin II gene.

This study was funded by the French Institute for Public Health Surveillance (Santé Publique France, grant CNR Paludisme). F.F.T. was supported by the Foundation Méditerranée Infection.

We declare that we have no competing interests.

The members of the French National Reference Centre for Imported Malaria Study Group are as follows: D. Basset (Centre Hospitalier Universitaire de Montpellier, Montpellier), P. Bastien (Centre Hospitalier Universitaire de Montpellier, Montpellier), F. Benoit-Vical (Centre Hospitalier Universitaire de Rangueil, Toulouse), A. Berry (Centre Hospitalier Universitaire de Rangueil, Toulouse), P. Brouqui (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), M. Cividin (Centre Hospitalier du Pays d'Aix, Aix en Provence), P. Delaunay (Centre Hospitalier Universitaire de l'Archet, Nice), L. Delhaes (Hôpital Pellegrin, Bordeaux), M. Drancourt (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), T. Gaillard (Hôpital d'Instruction des Armées Saint-Anne, Toulon), A. Genin (Centre Hospitalier du Pays d'Aix, Aix en Provence), E. Garnotel (Hôpital d'Instruction des Armées Laveran, Marseille), E. Javelle (Hôpital d'Instruction des Armées Laveran, Marseille), C. L'Ollivier (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), M. Leveque (Centre Hospitalier Universitaire de Montpellier, Montpellier), D. Malvy (Hôpital Pellegrin, Bordeaux), P. Marty (Centre Hospitalier Universitaire de l'Archet, Nice), G. Ménard (Hôpital d'Instruction des Armées Saint-Anne, Toulon), P. Millet (Hôpital Pellegrin, Bordeaux), P. Minodier (Hôpital Nord, Marseille), A. Mottard (Hôpital de Fréjus-Saint Raphael, Fréjus), P. Parola (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), R. Piarroux (Hôpital la Timone, Marseille), C. Pomares-Estran (Centre Hospitalier Universitaire de l'Archet, Nice), M.-C. Receveur (Hôpital Pellegrin, Bordeaux), A. Robin (Centre Hospitalier du Pays d'Aix, Aix en Provence), E. Sappa (Centre Hospitalier du Pays d'Aix, Aix en Provence), H. Savini (Hôpital d'Instruction des Armées Laveran, Marseille), F. Simon (Hôpital d'Instruction des Armées Laveran, Marseille), Y. Sterkers (Centre Hospitalier Universitaire de Montpellier, Montpellier), C. Surcouf (Hôpital d'Instruction des Armées Laveran, Marseille), E. Varlet (Centre Hospitalier Universitaire de Montpellier, Montpellier), and A. Wolff (Hôpital d'Instruction des Armées Laveran, Marseille).

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